AD_____

Award Number: W81XWH-04-1-0849

TITLE: AR-NcoR Interaction as a Therapeutic Target for Prostate Cancer Prevention and Treatment

PRINCIPAL INVESTIGATOR: Steven P. Balk

CONTRACTING ORGANIZATION: Beth Israel Deaconess Medical Center
Boston, MA 02215

REPORT DATE: October 2006

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-10-2006	Annual	5 Sep 2005 – 4 Sep 2006

4. TITLE AND SUBTITLE

AR-NcoR Interaction as a Therapeutic Target for Prostate Cancer Prevention and Treatment

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0849

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Steven P. Balk

E-Mail: sbalk@bidmc.harvard.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Beth Israel Deaconess Medical Center
Boston, MA 02215

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES
Original contains colored plates: ALL DTIC reproductions will be in black and white.

14. ABSTRACT

Aim 1 is to determine the precise molecular basis for NCoR binding to the RU486 liganded AR. Since the previous report we have used chromatin immunoprecipitation to demonstrate that RU486 enahnces AR NCoR recruitment to AR assembled on androgen regulated genes. We have also generated the additional AR and NCoR mutants to define the precise amino acids mediating the interaction. Aim 2 is to determine whether NCoR recruitment can suppress androgen independent expression of AR regulated genes and prostate cancer growth, and identify molecular markers that predict whether RU486 (or related drugs) will be effective in particular prostate cancers in vivo. We have now used chromatin immunoprecipitation to examine the functional effects of RU486 mediated NCoR recruitment, and find that NCoR is not mediating deacetylation and hence not suppressing gene expression. The reason for this is now under investigation. These results, in conjunction with our previous data, reflect further progress towards determining the structural basis for AR-NCoR interaction (Aim 1) and determining whether this interaction can be exploited to treat prostate cancer (Aim 2).

15. SUBJECT TERMS
ANDROGEN RECEPTOR, NCOR, STEROID HORMONE, ANTAGONIST, TRANSCRIPTION

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	8	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

INRODUCTION

The androgen receptor (AR) plays a central role in prostate cancer (PCa) and androgen ablation therapy is the standard systemic therapy for metastatic PCa, but most patients relapse with an aggressive stage of the disease termed hormone refractory or androgen independent PCa. The AR and androgen regulated genes are still expressed in androgen independent PCa, indicating that the AR remains as a therapeutic target for higher affinity pure antagonists. However, such drugs that can compete with dihydrotestosterone (DHT) for AR binding have not been developed. An alternative is the development of drugs that enhance AR recruitment of nuclear receptor corepressors (NCoR or SMRT), as such drugs could actively repress AR regulated genes. We have shown that the DHT liganded AR binds NCoR, and that this binding can be markedly enhanced by RU486 (mifepristone), a steroidal antagonist of the progesterone and glucocorticoid receptors. **Our hypothesis is that enhancement of the AR-NCoR interaction is a therapeutic approach for the treatment of PCa, including advanced androgen independent PCa.** The RU486 data provide a "proof of principle" that the AR-NCoR interaction can be enhanced, and suggest a novel mechanism for antagonist binding that may be valuable in the further development of high affinity AR antagonists**.**

Aim 1 is to determine the precise molecular basis for NCoR binding to the RU486 (mifepristone) liganded AR.

Aim 2 is to test the hypothesis that NCoR recruitment can suppress androgen independent expression of AR regulated genes and prostate cancer growth, and identify molecular markers that predict whether RU486 (or related drugs) will be effective in particular prostate cancers *in vivo.*

BODY

We present below our progress toward the specific aims that has occurred since the previous progress report.

Mifepristone enhances AR-NCoR binding as assessed directly by coimmunoprecipitation of endogenous proteins from LNCaP cells. Our published data demonstrating mifepristone enhancement of AR-NCoR binding were based primarily on functional assays and GST-pulldowns. Therefore, we determined whether mifepristone would enhance binding of endogenous AR and NCoR. LNCaP PCa cells grown in medium with 10% FBS were treated for 8 hrs with mifepristone and/or LY294002. The LY294002 treatment was based on preliminary data showing that PI3 kinase blockade in PTEN deficient LNCaP cells could increase NCoR levels (data not shown). Lysates were then immunoprecipitated with anti-AR (AR) or control (C) Abs and immunoblotted for NCoR. A weak interaction was detected in the absence of mifepristone (RU486), which was increased with mifepristone treatment alone or with LY294002, providing further direct evidence of enhanced AR-NCoR binding (Fig. 1.).

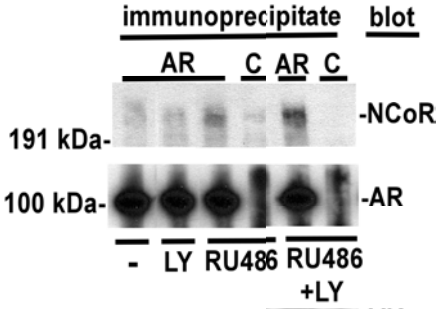

Fig. 1. Mifepristone (RU486) enhances coimmunoprecipitation of endogenous AR and NCoR. Confluent 10 cm plates of LNCaP cells were treated as indicated for 8 hours, lysed in TBS/1% Triton X-100/protease inhibitors, and precleared with nonimmune Ab on protein A and G beads. Lysates were split for specific precipitation using a pool of rabbit anti-AR N and C-terminal Abs (Santa Cruz) and mAb clone 441 against an epitope in the middle of the N-terminus, with the control using equal amounts of irrelevant Ab. Eluted proteins were run on 4-12% gradient gels and blotted with anti-NCoR or anti-AR.

Mifepristone mediates NCoR recruitment to the *PSA* locus in C4-2 cells. The PSA gene has been used extensively as a model for AR mediated transcription. As shown in figure 2A, it contains a consensus ARE in the promoter (ARE I) and a strong consensus ARE in the enhancer (ARE III), which is flanked by a series of weak AREs. Control chromatin immunoprecipitation (ChIP) experiments in COS7 cells using transfected AR and an episomal luciferase reporter gene regulated by the PSA promoter and enhancer (extending to ~6 Kb upstream of the transcriptional start site, pPSA-luciferase) showed that mifepristone stimulated AR recruitment and strongly stimulated NCoR recruitment (relative to DHT) to ARE I and ARE III (Fig. 2B). Significantly, NCoR recruitment to ARE I and III in the endogenous PSA gene was also strongly stimulated by mifepristone in C4-2 cells (Fig. 2B).

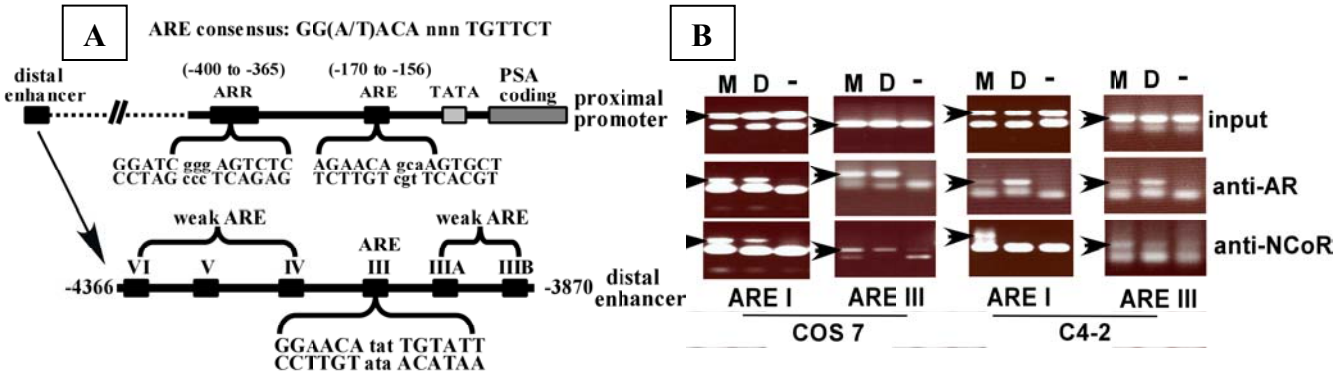

Fig. 2. AR and NCoR recruitment to the PSA locus by ChIP. A, outline of PSA locus. B, COS 7 cells transfected with pPSA-luciferase and AR or C4-2 cells (not transfected) were cultured in steroid hormone depleted medium and then stimulated for 2 hours with 1 μM mifepristone (M), 10 nM DHT (D), or vehicle (-). Cells were then formalin fixed and ChIP was done with anti-AR or anti-NCoR. Arrows indicate the ARE bands.

RU486 mediates increased NCoR recruitment to the wild-type AR relative to DHT or bicalutamide. We next wanted to confirm that RU486 mediated recruitment of NCoR was enhanced relative to DHT or bicalutamide, using the wild-type AR (LNCaP and C4-2 cells have a T877A mutant AR that affects the response to ligand binding). One impediment has been the lack of prostate cancer cell lines expressing wild-type AR that can be induced by androgen to express androgen regulated genes (as the generally available LNCaP and CWR22 derived cells have AR mutations that diminish mifepristone mediated AR-NCoR binding, while the LAPC-4 cell line produces minimal PSA in response to androgen stimulation). Therefore, we generated stable PC3 cells (AR negative prostate cancer cells) expressing wild-type AR and an integrated androgen responsive PSA reporter gene. We have now successfully screened a series of stable PC3 clones transfected with wild-type AR and containing an integrated PSA-luciferase reporter gene, and identified clones with androgen stimulated luciferase production (Fig. 3).

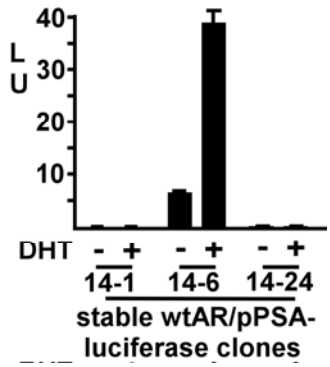

Fig. 3. PC3 cells with stable AR and reporter expression. PC3 cells were transfected with a wild-type AR expression vector and a luciferase reporter regulated by the PSA gene 5' upstream region (~6 kilobases, including the major enhancer ARE III). Stable transfectants were drug selected and clones were then screened for DHT stimulated luciferase synthesis. Clone 14-6 showed basal luciferase activity in steroid hormone depleted medium, which was enhanced ~7-fold by 24 hour treatment with 10 nM DHT.

With these cells in hand, we have been able to directly test the hypothesis that mifepristone can enhance NCoR recruitment to androgen regulated genes. We have also directly compared recruitment mediated by the T877A mutant AR (in LNCaP and C4-2 cells) versus the wild-type AR. In the experiment shown in figure 4, the above 14-6 PC3 cell clone and C4-2 cells were cultured in steroid hormone depleted medium and then stimulated for 48 hours with mifepristone or DHT (both at 100 nM), or bicalutamide (10 μM). Chromatin immunoprecipitations (ChIP) were then carried out with anti-AR and anti-NCoR antibodies, and binding to ARE III in the PSA gene was assessed by PCR. As shown in figure 4, AR binding to the ARE III in C4-2 cells was strongly stimulated by DHT, and weakly stimulated by mifepristone and bicalutamide. NCoR binding was also detected in response to bicalutamide and DHT, but was substantially enhanced by mifepristone. The results with the PC3 cells expressing wild-type AR were consistent with the C4-2 cells, but indicated more robust mifepristone mediated NCoR recruitment. In particular, mifepristone mediated recruitment of NCoR was markedly enhanced relative to AR, which was only weakly associated after 48 hours of mifepristone.

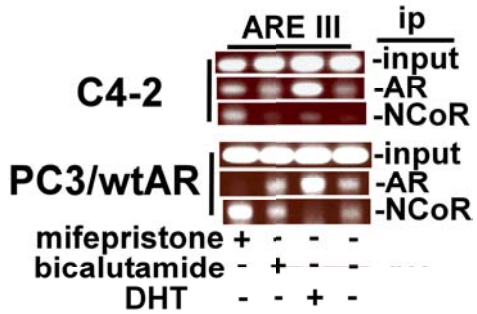

Fig. 4. ChIP analysis of C4-2 and PC3/AR cells. C4-2 cells or stable PC3 transfectants with wild-type AR and integrated PSA regulated reporter (clone 14-6) were treated for 48 hours as indicated. Anti-AR and anti-NCoR ChIP was then performed and binding to the ARE III in the PSA gene enhancer was assessed by PCR amplification.

Basal expression of AR regulated genes in C4-2 cells is not suppressed by bicalutamide or mifepristone. Significantly, although mifepristone mediated NCoR recruitment to the PSA gene in C4-2 cells, the basal expression of PSA (in steroid hormone depleted medium) was not suppressed by bicalutamide or mifepristone, with mifepristone instead having weak partial agonist activity (Fig. 5). Similarly, these drugs did not suppress basal expression of other AR regulated genes. We have therefore focused on the functional activity of NCoR recruited by bicalutamide and mifepristone, and in particular whether it can stimulate significant levels of histone deacetylation.

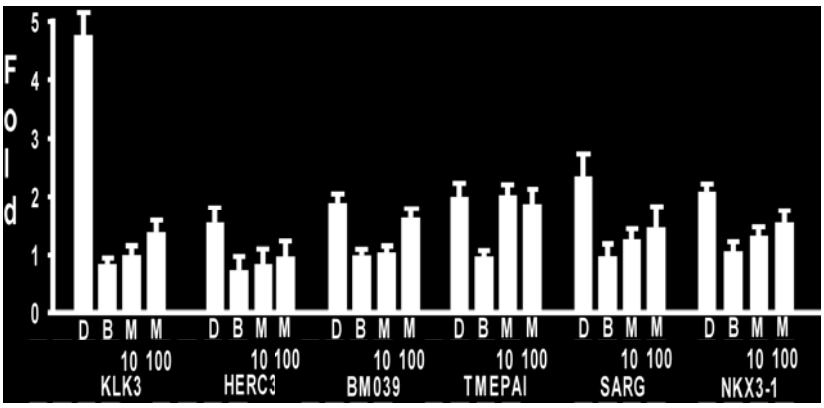

Fig. 5. Effects of AR antagonists on basal expression of PSA (KLK3) and other AR regulated genes in C4-2 cells. C4-2 cells cultured in steroid hormone depleted medium for 3 days were stimulated for 24 hours with 1 nM DHT (D), 10 μM bicalutamide, or mifepristone (M) at 10 or 100 nM. Message levels of PSA (KLK3) and other genes were then measured and expressed as fold change compared to vehicle treated cells

Our interpretation of these data is that while the mifepristone liganded AR mediates the recruitment of NCoR, this either occurs on a minority of sites or it fails to co-recruit histone deacetylase activiity. To test this hypothesis using cells with an endogenous wild-type AR, we

have used LAPC-4 cells. As observed above, mifepristone was able to markedly enhance NCoR recruitment to ARE III in the PSA gene relative to DHT and bicalutamide (Fig. 6). However, we did not observe the anticipated decrease in histone acetylation at ARE III, as assessed by ChIP using anti-acetyl-lysine antibodies, although a decrease was observed at ARE I (Fig. 6). Studies are now underway to determine why the recruited NCoR is not mediating further histone deacetylation, and whether that may be enhanced to suppress gene expression. Overall, we believe these results demonstrate that the AR-NCoR protein-protein interaction mediated by mifepristone translates into enhanced NCoR recruitment to androgen regulated genes, and support the overall feasibility targeting the AR-NCoR interaction for prostate cancer therapy.

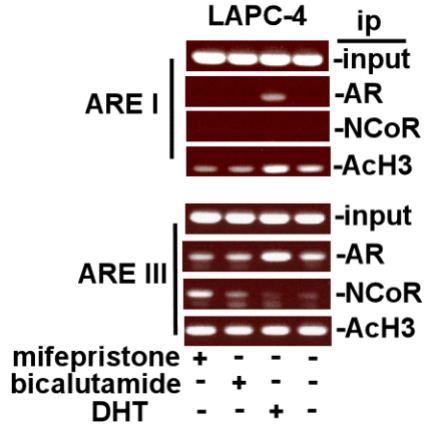

Fig. 6. ChIP analysis of LAPC-4 cells. LAPC-4 cells (which express a wild-type AR) were treated for 48 hours as indicated (mifepristone and DHT at 100 nM, and bicalutamide at 10 μM). Anti-AR, anti-NCoR, and anti-hisotne 3 acetyl-lysine ChIP was then performed and binding to the ARE I and ARE III in the PSA gene was assessed by PCR amplification.

Clinical trial of mifepristone in androgen independent PCa. Based on the novel properties of mifepristone as an AR antagonist, we conducted a small phase II trial of this drug in men with androgen independent PCa. The PI was M. Taplin (Dana Farber), with co-investigators S. Balk and E. Gelmann (Georgetown). This study was not supported by this DOD grant, but is included here as it is clearly relevant to the objectives of the proposal. A total of 19 patients with rising PSA (≥5 ng/mL, median 36.9 ng/mL, range 5.2-1,931 ng/mL) and documented bone metastases were treated with mifepristone (200 mg PO QD) until disease progression. Anticipating a possible increase in adrenal hormone production due to glucocorticoid receptor blockade, we included correlative studies of serum testosterone, DHT, 3α-diol glucuronide (a DHT metabolite), androstenedione, and DHEA-sulfate levels at baseline and during therapy. No patients demonstrated a PSA response, as defined by a >50% decrease in baseline PSA. However, this result was confounded by marked and highly significant increases in all hormones measured: DHEA-sulfate 2.8 fold, androstenedione 1.8 fold, testosterone 4.0 fold, DHT 1.5 fold, and 3α-diol glucuronide 1.9 fold. Significantly, 6 patients (30%) had stable disease by PSA criteria for ≥ 5 months, and this correlated with less stimulation of all hormones tested, with the difference in androstenedione (0.95 ng/mL versus 2.27 ng/mL) reaching statistical significance (p=0.032). These data indicate that mifepristone may have AR antagonist activity in a subset of androgen independent PCa patients, and a subsequent trial is under consideration that would include dexamethasone to prevent the increase in adrenal androgen production. However, the long-term goal is clearly to obtain a more selective and potent AR antagonist.

KEY RESEARCH ACCOMPLISHMENTS (1-4 from previous report and 5-7 from this report)

1. Demostrated that AR N-terminus is critical for AR-NCoR interaction.
2. Demostrated that lysine 720 in AR LBD is critical for binding.
3. Identified the N1 CoRNR box as mediating binding to the AR LBD.

4. Demonstrated that RU486 could suppress the androgen independent C4-2 prostate cancer cell line.
5. Confirmed that RU486 enhances binding of endogenous AR and NCoR
6. Confirmed that RU486 enhances recruitment of endogenous NCoR to ARE III in the the endogenous *PSA* gene in prostate cancer cells.
7. Examined effects of NCoR recruitment on gene expression and histone acetylation.

REPORTABLE OUTCOMES
One manuscript published (attached to previous report) (Hodgson et al., 2005)
Two new manuscripts in preparation.

CONCLUSIONS
We have made substantial progress towards determining the structural basis for AR-NCoR interaction (Aim 1) and determining whether this interaction can be exploited to treat prostate cancer (Aim 2).

REFERENCES
Hodgson,M.C., Astapova,I., Cheng,S., Lee,L.J., Verhoeven,M.C., Choi,E., Balk,S.P., and Hollenberg,A.N. (2005). The androgen receptor recruits nuclear receptor CoRepressor (N-CoR) in the presence of mifepristone via its N and C termini revealing a novel molecular mechanism for androgen receptor antagonists. J. Biol. Chem. *280*, 6511-6519.

APPENDICES
none